Exhibit 5

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GENES V

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CHAPTER 21

The extraordinary power of DNA technology

The technology for dealing with DNA has become so powerful that it is now a routine project to obtain the DNA corresponding to any particular gene. At the heart of this technology is the ability to amplify individual DNA sequences. **Cloning** a fragment of DNA allows indefinite amounts to be produced from even a single original molecule. (A **clone** is defined as a large number of cells or molecules all identical with an original ancestral cell or molecule.) Once any particular segment of DNA has been cloned, its properties can be characterized. Its sequence should reveal whether it is likely to code for a protein; and the cloned segment (or parts of it) can be used to test whether it contains sites that are bound by regulatory proteins.

Cloning technology involves the construction of novel DNA molecules by joining sequences from different sources. The product is often described as **recombinant DNA**, and the techniques (more colloquially) as **genetic engineering**. They are applicable equally to prokaryotes and eukaryotes, although the power of this approach is especially evident with eukaryotic genomes.

Cloning of DNA is made possible by the ability of bacterial plasmids and phages to reproduce after additional sequences of DNA have been incorporated into their genomes. An insertion generates a **hybrid** or **chimeric** plasmid or phage, consisting in part of the authentic DNA of the original genome and in part of the additional 'foreign' sequences. These chimeric elements replicate in bacteria just like the original plasmid or phage and so can be obtained in large amounts. Copies of the original foreign fragment can be retrieved from the progeny. Since the properties of the chimeric species usually are unaffected by the particular foreign sequences that are involved, almost any sequence of DNA can be cloned in this way. Because the phage or plasmid is used to 'carry' the foreign DNA as an inert part of the genome, it is often referred to as the **cloning vector**.

Cloning a specific gene requires the ability to identify or characterize particular regions or sequences of the genome. In practical terms, we need a **probe** that will react with the target DNA. If a gene has a known product, in principle it is possible to work back from the protein to the gene, by obtaining the mRNA that codes for the protein and using it (directly or indirectly) as a probe to isolate the gene.

Practical questions are therefore first how to identify the RNA (from the cytoplasm) that represents a particular gene, and then how to obtain the DNA (from the genome) that codes for the RNA. Having identified this nucleic acid, how do we obtain a sufficient amount of material to characterize? Can we direct the isolated coding sequence to synthesize its product *in vitro* or *in vivo*? Can we introduce changes in this sequence that will influence its expression and help reveal the nature of regulatory signals?

One major concern is the need to characterize genes whose products are unknown. When a gene is thought to be expressed in one cell type but not

in another, closely related cell, we can attempt to identify those mRNAs found only in one of the cells. In practice, this involves 'subtracting' one mRNA population from the other to find the mRNAs that are unique to one cell.

Another common problem lies with human diseases that are caused by known genetic traits, but where the gene product (and sometimes even the original malfunctioning cell type) is not known. By genetic analysis the chromosome conveying the genetic trait is identified; then the gene is tracked to a region of the chromosome by genetic characterization of individuals with the disease, and finally we begin to search at the molecular level for a gene within this region that can be associated with the disease. But it is not a trivial problem to identify the correct gene in a large region when the molecular nature of the disease is not well defined.

As well as leading to the isolation of the DNA for genes of interest, cloning technology has significant implications for diagnostic procedures. Once we know that a certain sequence is associated with a particular allele, it is possible to test any individual for the presence of the sequence. In principle, this allows the genotype of an individual to be determined directly for any trait of importance.

Any DNA sequence can be cloned in bacteria or yeast

Hybrid DNA molecules are constructed by using restriction enzymes to cleave DNA at particular, rather short nucleotide sequences (see Chapters 6 and 20). By cleaving both the vector and the target DNA at appropriate sites, we can rejoin them to construct hybrid molecules that can be used to amplify the amount of material or to express a particular sequence.

A critical feature of any cloning vector is that it should possess a site at which foreign DNA can be inserted without disrupting any essential vector function. The simplest approach is to use a restriction enzyme that has only a single target site, at a nonessential location in the vector DNA. The insertion procedure generates only a small proportion of chimeric genomes from the starting material, so it is important to have some means of *selecting* the chimeric genome from the original vector.

Plasmid genomes are circular, so a single cleavage converts the DNA into a linear molecule, as depicted in **Figure 21.1**. Then the two ends can be joined to the ends of a linear foreign DNA, regenerating a circular chimeric plasmid. The length of foreign DNA that can be inserted is limited only by practical considerations, such as the susceptibility of long DNA molecules to breakage. The chimeric plasmid can be perpetuated indefinitely in bacteria. It can be isolated by virtue of its size or circularity—for example, by gel electrophoresis.

Many plasmids carry genes that specify resistance to antibiotics. This feature is useful in designing cloning systems. A common procedure is to use a plasmid that has genes specifying resistance to two antibiotics. One of the genes is used to identify bacteria that carry the plasmid. The other is used to distinguish chimeric plasmids from parental vectors. If the site used to insert foreign DNA lies within this second gene, the chimeric plasmid *loses* the antibiotic resistance. Thus a parental vector can be identified by its resistance to both antibiotics; and a chimeric plasmid can be selected by its retention of resistance to one antibiotic, but sensitivity to the other.

(As a practical matter, bacteria that are being tested for sensitivities to antibiotics or other agents are maintained by **replica plating**. Replicas made

from a master isolate are tested for sensitivity; if they are killed by the selective agent, the strain can be retrieved from the master, as shown later in Figure 21.11.)

Cloning vectors that have all the desired properties have been developed by making improvements to naturally occurring plasmids. This manipulation may involve the introduction of changes in the replication control system or the addition of genes determining resistance to particular antibiotics. One of the classic cloning vectors is **pBR322**, which was derived by several sequential alterations of earlier cloning vectors. It is a multicopy plasmid carrying genes for resistance to tetracycline and ampicillin; several restriction enzymes have unique cleavage sites at useful locations.

Phages provide another type of vector system.

Figure 21.1

Overview: plasmid vectors can be used to clone any fragment of DNA that is inserted at an appropriate site.

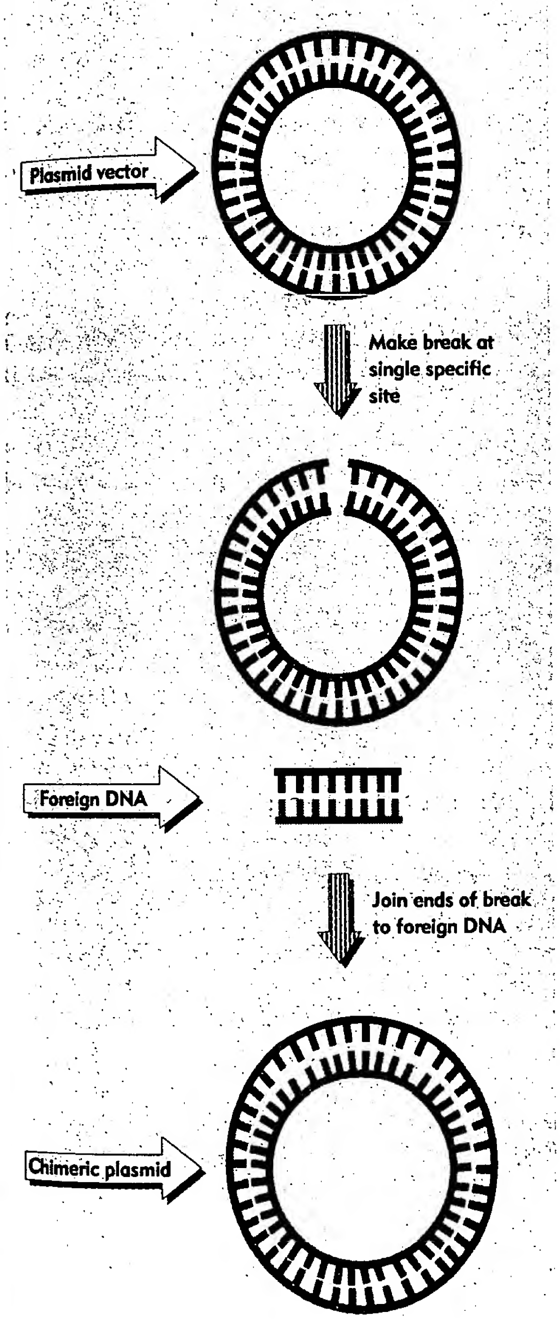
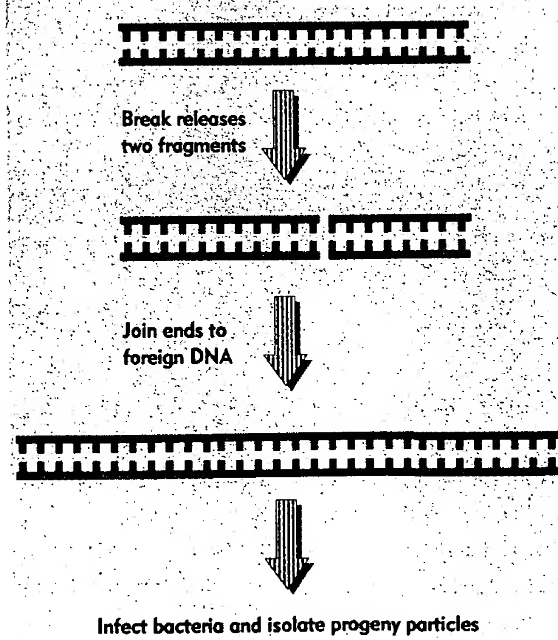


Figure 21.2

Phage vectors can be used to clone a foreign DNA that is inserted into a nonessential region of the genome.



Usually the phage is a linear DNA molecule, so that a single restriction break generates two fragments. They are joined together with the foreign DNA to generate a chimeric phage as shown in **Figure 21.2**. Chimeric phage genomes can be conveniently isolated by allowing the phage to proceed through the lytic cycle to produce particles. However, this procedure imposes a limit on the length of foreign DNA that can be cloned, because the capacity of the phage head prevents genomes that are too long from being packaged into progeny particles.

To ameliorate this problem, a fragment of the vector that does not carry any essential phage genes can be *replaced* by the foreign DNA. This approach has been taken to a fine art with phage lambda, where a new vector has been created by manipulating the DNA to produce a shorter genome (lacking nonessential genes) that actually is *too short to be packaged into the phage head*, which has a minimum as well as maximum length requirement. Thus it is *necessary* for a foreign DNA fragment to be inserted into the cleaved parental vector in order to generate a phage that can be perpetuated as progeny particles. This demand creates an automatic selective system for obtaining chimeric phage genomes (with inserted DNA of the right length).

The utility of this type of vector has been increased by the development of systems for packaging the DNA into the phage particle *in vitro*. An attempt to combine some of the advantages of plasmids and phages led to the construction of **cosmids**. These are plasmids into which have been inserted the particular DNA sequences (*cos* sites) needed to package lambda DNA into its particle. These vectors still can be perpetuated in bacteria in the plasmid form, but can be purified by packaging *in vitro* into phages. They are still subject to the length limitation imposed by the particle head, but more of the foreign DNA can be packaged since phage genes are not needed.

We have dealt with cloning vectors in the context of using bacterial hosts. Sometimes it is useful to use a eukaryotic host. There are few authentic eukaryotic plasmids: the yeast 2μ plasmid and BPV (bovine papilloma virus) are two that have been characterized. By reconstruction of DNA, some 'dual-purpose' or 'shuttle' plasmids have been obtained that have the necessary sequences for surviving in either *E. coli* or *S. cerevisiae*. Thus the one vector can be used with either host.

In dealing with higher eukaryotic genomes, it is often necessary to handle very large fragments of DNA. We see later that this can be accomplished by the use of YACs (yeast artificial chromosomes), in which DNA fragments of ~300 kb can be propagated in yeast.

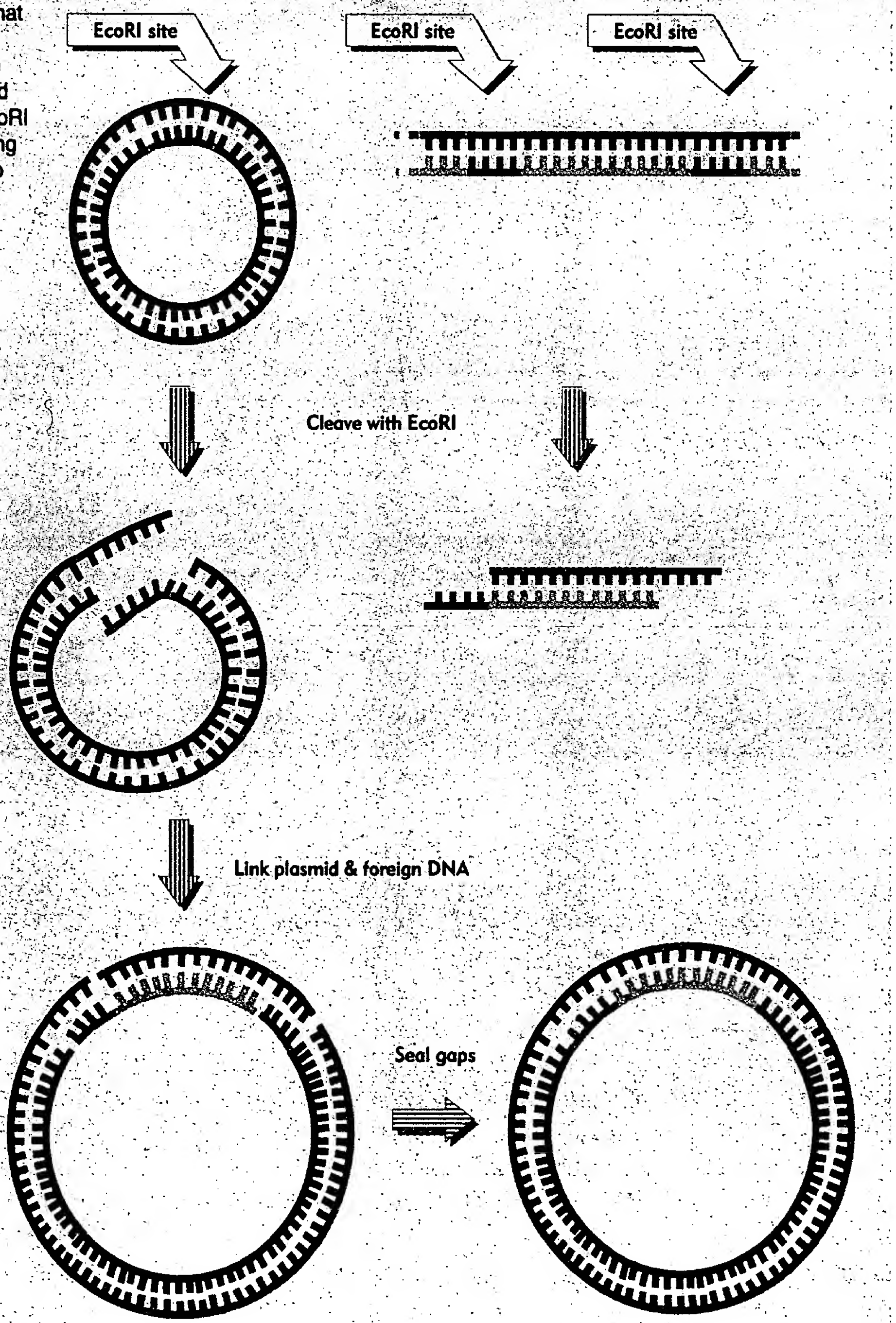
Constructing the chimeric DNA

To join a foreign DNA fragment to a cloning vector requires a reaction between the ends of the fragment and the vector. This can be accomplished by generating complementary sequences on the fragment and vector, so that they can recombine into a chimeric DNA when mixed together.

The most common method is to use restriction enzymes that make **staggered** cuts to generate short, complementary single-stranded **sticky ends**. The classic example is provided by the enzyme *Eco*RI, which cleaves each of the two strands of duplex DNA at a different point. These sites lie on

Figure 21.3

Any DNA sequence that lies between EcoRI cleavage sites can be inserted into a plasmid vector that has an EcoRI cleavage site by cutting and annealing the two DNA molecules.



either side of a short palindromic sequence that is part of the site recognized by the enzyme.

The *Eco*RI recognition sequence consists of 6 bases (highlighted in color), and the enzyme cuts at the bonds indicated by the vertical arrows:

↓
5′ ...pNpNpNpGpApApTpTpCpNpNpN ...3′
3′ ... NpNpNpCpTpTpApApGpNpNpNp...5′
↑

The DNA fragments on either side of the target site fall apart, and because of the stagger of individual cutting sites, they have protruding single-stranded regions that are complementary—the sticky ends indicated by the color:

pApApTpTpCpNpNpN ...3′
GpNpNpNp...5′

and

5′...pNpNpNpG
3′... NpNpNpCp TpTpApAp

Their complementarity allows the sticky ends to anneal by base pairing. When two different molecules are cleaved with EcoRI, the *same* sticky ends are generated on both molecules. This enables one to anneal with the other, as illustrated in the protocol of **Figure 21.3**. The procedure generates a chimeric plasmid that is intact except for the lack of covalent bonds between the vector and the foreign DNA. The missing bonds are made good by the enzyme DNA ligase. This technique for recombining two DNA molecules has both *pros* and *cons*.

An advantage is that the chimeric plasmid possesses regenerated *Eco*RI sites at either end of the inserted DNA. Thus the foreign DNA fragment can be retrieved rather easily from the cloned copies of the chimeric vector, just by cleaving with *Eco*RI.

A disadvantage is that any *Eco*RI sticky end can anneal with any other *Eco*RI sticky end. Thus some vectors reform by direct reaction between their ends, without gaining an insert, while others gain an insert of several foreign fragments joined end to end. To avoid such complications, it is therefore necessary to select chimeric plasmids that have gained only a single insert.

A problem in relying exclusively on restriction enzymes to generate ends for the joining reaction is that their recognition sites may not lie at convenient points in the foreign DNA sequence. Another method allows any DNA end to be used for recombination with a plasmid.

The plasmid is cleaved as before with an enzyme that recognizes a single site in a suitable location, but this need not necessarily generate staggered ends. The enzyme **terminal transferase** is used with the precursor dATP to add a stretch of polyadenylic acid [poly(dA)] to both the 3′ ends of the plasmid DNA. In the same way, poly(dT) is added to the 3′ ends of the foreign DNA molecule that is to be inserted; these ends also can have been generated in any convenient manner.

As **Figure 21.4** demonstrates, the poly(dA) on the plasmid can anneal *only* with the poly(dT) on the insert fragment. Thus only one reaction is possible: the insertion of a single foreign fragment into the vector.

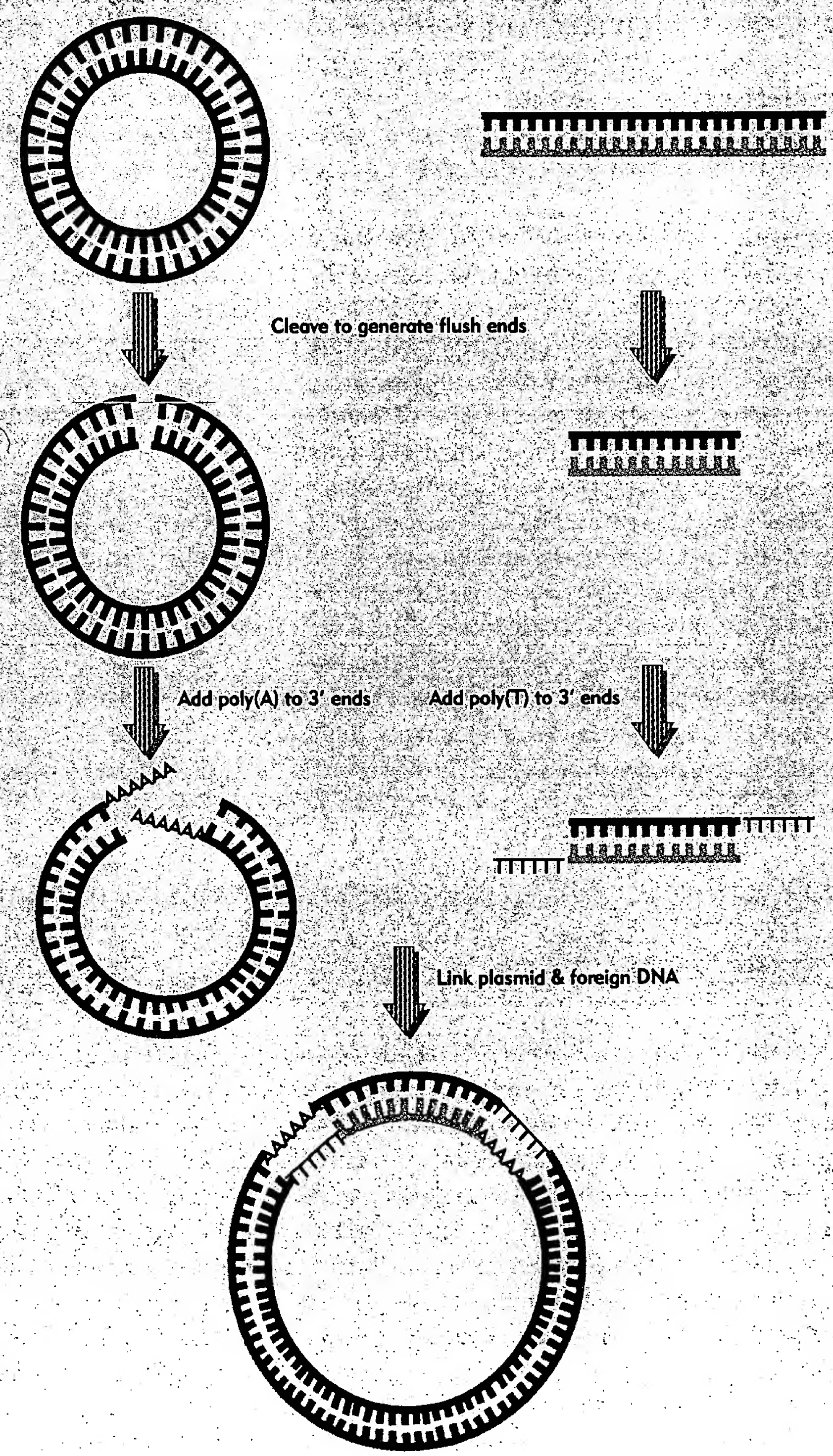
A drawback of this technique is that it is not so easy to retrieve the inserted fragment from the cloned chimeric plasmid, because the recognition site for the restriction enzyme in the original vector has been abolished by the insertion of the foreign DNA. The inserted sequence is flanked on either side by the poly(dA) : poly(dT) paired region.

Another useful technique is **blunt-end ligation** which relies on the ability of the T4 DNA ligase to join together two DNA molecules that have blunt ends, that is, they lack any protruding single strands. (This reaction is in addition to the usual activity of joining broken bonds within a duplex.) When DNA has been cleaved with restriction enzymes that cut across both strands at the same position, blunt-end ligation can be used to join the fragments directly together.

The great advantage of this technique is that any pair of ends may be joined together, irrespective of sequence. This is especially useful when we want to join two defined sequences without introducin

Figure 21.4

The poly(dA:dT) tailing technique allows any two DNA molecules to be joined by adding poly(dA) to the 3' ends of one and adding poly(dT) to the 3' ends of the other.



any additional material between them. A problem inherent in this technique is that there is no control over which pairs of blunt ends are joined together, so it is necessary first to perform the reaction and then to isolate the desired products from among the other products.

There are numerous variations of these methods. One technique uses short DNA duplexes ('linkers') that contain the *Eco*RI (or some equivalent) recognition palindrome. The linkers can be synthesized chemically, and are added covalently to the ends of a plasmid or an insert by blunt-end ligation. The inserted DNA can be retrieved by cleavage with *Eco*RI, but there are no restrictions on the original choice of sites to generate the ends. With sufficient manipulation, it is now possible to insert any foreign DNA fragment into any particular vector site, and to arrange for retrieval of the fragment when necessary.

When a foreign DNA fragment is inserted into a plasmid, it can be connected in either orientation, that is, with either of the ends of the foreign DNA joined to either of the ends of the plasmid. This does not matter when the purpose of cloning is simply to amplify the inserted sequence. However, it is important when the experiment is designed to obtain expression of the foreign DNA, which requires insertion in a particular orientation.

In this case, populations of plasmids carrying the plasmid in either orientation are obtained via random insertion, after which they are characterized by restriction mapping to identify the desired class. Or the experiment is designed so as to permit insertion in one orientation only. For example, each of the DNAs, vector and insert, may be cleaved with *two* restriction enzymes that make different sticky ends, to generate the type of pattern where each DNA has the sequence

```
End 1 ————————————End 2
```

Now if only the two end-1 sequences can anneal together, and only the two end-2 sequences can anneal, the insertion can take place only in one orientation, generating the chimeric plasmid

```
End 1———Insert———End 2
  |                 |
End 1———Plasmid———End 2
```

Copying mRNA into cDNA

One of the principal uses of cloning technology is to isolate specific genes directly from the genome. Any particular gene represents only a very small part of a eukaryotic genome. In a typical mammal, the size of the genome is ~10^9 bp, so that a single gene of (say) 5000 bp represents only 0.0005% of the total nuclear DNA.

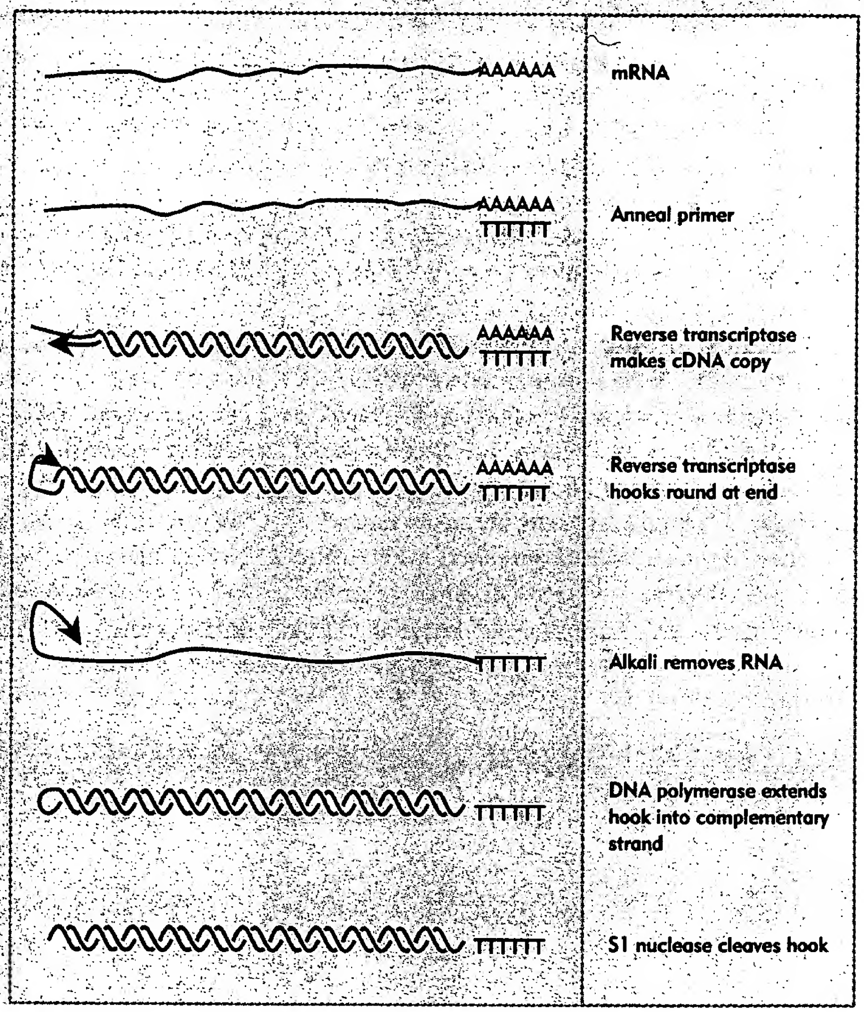
To identify such a tiny proportion, we need a very specific probe that reacts *only* with the particular sequence in which we are interested, to pick it out from the vast excess of other sequences. The usual technique is to use a highly labeled radioactive probe of RNA or DNA, whose hybridization with the gene is assayed by autoradiography.

For the purpose of obtaining a DNA sequence that represents a particular protein, the place to start is with mRNA, which, after all, is the template used to produce the protein *in vivo*. But it can be difficult to obtain the mRNA that represents a particular protein when the product is rare. There are several techniques for isolating an mRNA via the properties of its product, but a common problem is the requirement that the RNA must first be purified.

Rather than purify the RNA, a DNA copy of the RNA sequence is made. This has the twin advantages that unlimited amounts of material can be obtained, and the DNA can be radioactively

Figure 21.5

mRNA can be copied into double-stranded DNA.



labeled, providing a much more powerful probe. The existence of reverse transcription makes it possible to synthesize a duplex DNA from any mRNA. This is especially easy for mRNAs that carry a poly(A) tail at the 3′ end, as illustrated in **Figure 21.5**.

First, a **primer** is annealed to the poly(dA). It is a short sequence of oligo(dT), whose purpose is to provide a free 3′ end that can be used for extension by the enzyme reverse transcriptase. The enzyme engages in the usual 5′–3′ elongation, adding deoxynucleotides one at a time, as directed by complementary base pairing with the mRNA template.

The product of the reaction is a hybrid molecule, consisting of a template RNA strand base-paired with the complementary DNA strand. The only practical problem is the propensity *in vitro* of reverse transcriptase to stop before it has reached the 5′ end of the mRNA. In this case, the resulting reverse transcript falls short of representing the entire mRNA, because it lacks some of the sequences complementary to the 5′ end. However, by judicious adjustment of the experimental conditions, usually it is possible to persuade reverse transcriptase to proceed all the way.

A useful reaction tends to occur at the end of the mRNA, where the enzyme causes the reverse

transcript to 'loop back' on itself, by using the last few bases of the reverse transcript as a template for synthesis of a complement. That is, the end of the complementary DNA is used to direct synthesis of a short sequence that is identical with the mRNA, and which displaces it. This creates a short hairpin, usually 10–20 bp long.

At this juncture, the original mRNA is degraded by treatment with alkali (a procedure that does not affect DNA). The product is a single-stranded DNA that is complementary to the mRNA; it is called **cDNA**.

The hairpin at the 3′ end of the cDNA provides a natural primer for the next step, the use of *E. coli* DNA polymerase I to convert the single-stranded cDNA into a duplex DNA via synthesis of the complementary strand. In this reaction, the enzyme uses the cDNA as template for synthesis of a sequence identical with the original mRNA. The product is a duplex molecule with a hairpin at one end. The hairpin is cut by the enzyme S1 nuclease (which specifically degrades single-stranded DNA) to generate a conventional DNA duplex.

The duplex DNA can be cloned to generate large amounts of a synthetic gene representing the mRNA sequence. This is called a **cDNA clone**. (From this terminology, a somewhat looser use of the term 'cDNA' has emerged, being taken to describe the duplex insert and not just the original single-stranded reverse transcript.)

The power of sequencing technology has made it possible to bypass most of the problems posed by rare mRNAs by targeting a probe directly for the mRNA sequence. One powerful technique requires knowledge of only a small sequence of the protein. Short oligonucleotides can be synthesized tha correspond to this sequence. A variety of oligo nucleotides can be made to cover possible altern ative codons, especially at third base positions These oligonucleotides can be used to isolat cDNAs or genomic DNA that include the se quence of the corresponding gene.

Isolating individual genes from the genome

The first step toward identifying the gene corresponding to a particular probe is to break the DNA of the genome into fragments of a manageable size. It is desirable to obtain the gene in as few fragments as possible (ideally only one). Usually the maximum lengths of DNA that can be manipulated directly are in the range of 15–20 kb. Sometimes it is not possible to obtain a gene in the form of a single fragment, and then its structure must be determined by piecing together the information gained from its various fragments. (We have discussed the use of overlapping fragments in Chapter 6.)

The best technique for fragmenting a genome is to make a restriction digest. Then every fragment ends in a site that was recognized by that particular enzyme. However, restriction sites may occur at inconvenient locations—for example, in the middle of a gene that is to be cloned. One way to avoid this is to use more than one restriction enzyme, that is to repeat the experiment with different enzyme whose recognition sites lie at different location But this is time consuming; and when a lon sequence is involved, it may be difficult to find a enzyme that does not cleave within it.

When the DNA of an entire genome is digeste with a restriction enzyme, the frequency breakage is controlled by the length of th sequence recognized by the enzyme. The longe the sequence, the less often it occurs by chanc The probability that a particular 4 bp sequenc will occur is $0.25^4 = 1/256$, so that an enzym with such a short recognition sequence will cleav DNA rather frequently. The frequency decline to 1/1000 for a 5 bp sequence and to 1/4000 f a 6 bp sequence.

(This calculation assumes that each base

equally well represented in DNA, which usually is not the case. The frequency of cutting can be decreased by using an enzyme whose target sequence contains base pairs that are less predominant in the base composition of the DNA, and *vice versa*.)

To make a useful set of fragments from a restriction digest, a trick is employed to reduce the frequency of cutting. An enzyme with a short (4bp) recognition sequence is used under conditions that generate a *partial* digest. Any particular target site is cleaved only occasionally, so not all target sites are cleaved in any particular DNA molecule. The infrequent cleavage at each site, together with the frequent distribution of sites, means that the fragment distribution approaches a random cleavage of the genome. But each fragment ends in the same sequence, which can be chosen to contain a sticky end and is therefore useful for cloning.

The distribution of sites recognized by an enzyme becomes a matter of chance taken over the genome as a whole. So a restriction digest of eukaryotic DNA generates a continuum of fragments. When electrophoresed on a gel, these fragments form a smear in which no distinct bands are evident (with the exception of some repeated sequences, which are discussed in Chapter 26). However, when a specific probe is available, it is possible to detect the corresponding sequences in the restriction smear.

DNA fragments cannot be handled directly on an agarose gel. The key feature in the protocol for identifying fragments is the ability to transfer the DNA to a medium on which hybridization reactions can occur. So the DNA is denatured to give single-stranded fragments that are transferred from the agarose gel to a nitrocellulose filter on which they become immobilized. The procedure used to transfer the DNA is somewhat akin to blotting, and this is used colloquially as a description of the procedure. When performed with DNA, it is known as **Southern blotting** (named for the inventor of the procedure).

Figure 21.6 illustrates the protocol for transferring the DNA fragments. The agarose gel is placed on a filter paper that has been soaked in a concentrated salt solution. Then the nitrocellulose filter is placed on the gel, and some dry filter paper

Figure 21.6

Southern blotting transfers DNA fragments from an agarose gel to a nitrocellulose filter.

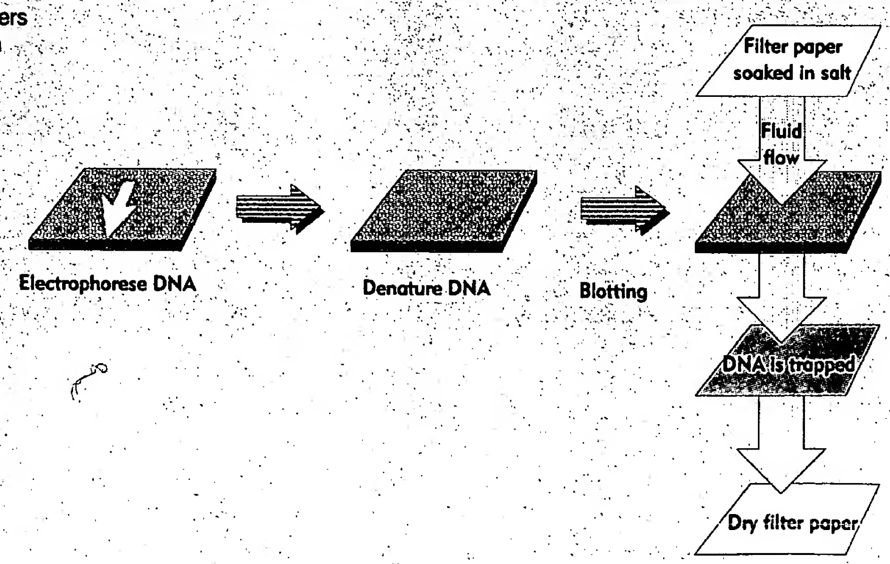
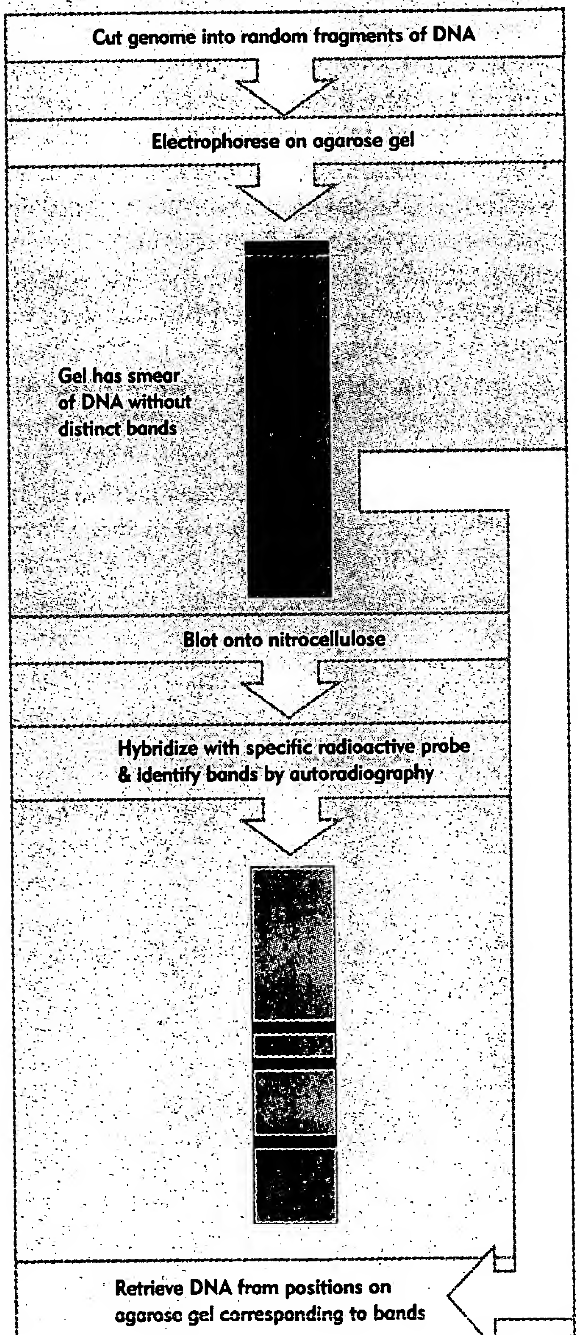


Figure 21.7

Southern blotting allows DNA fragments corresponding to a particular probe to be isolated directly from a digest of the DNA of the genome.



is placed in contact with the nitrocellulose. The salt solution is attracted to the dry filter paper. To get there, it must pass through the agarose gel and then through the nitrocellulose filter. The DNA is carried along with it, but becomes trapped in the nitrocellulose in the same relative position that it occupied in the gel.

DNA that has been immobilized on the nitrocellulose can be hybridized *in situ* with a radioactive probe, as indicated in **Figure 21.7**. Only those fragments complementary to a particular probe will hybridize with it. Because the probe is radioactive, the hybridization can be visualized by autoradiography. Each complementary sequence gives rise to a labeled band at a position determined by the size of the DNA fragment. Clearly the usefulness of this technique depends on the specificity of the available probes.

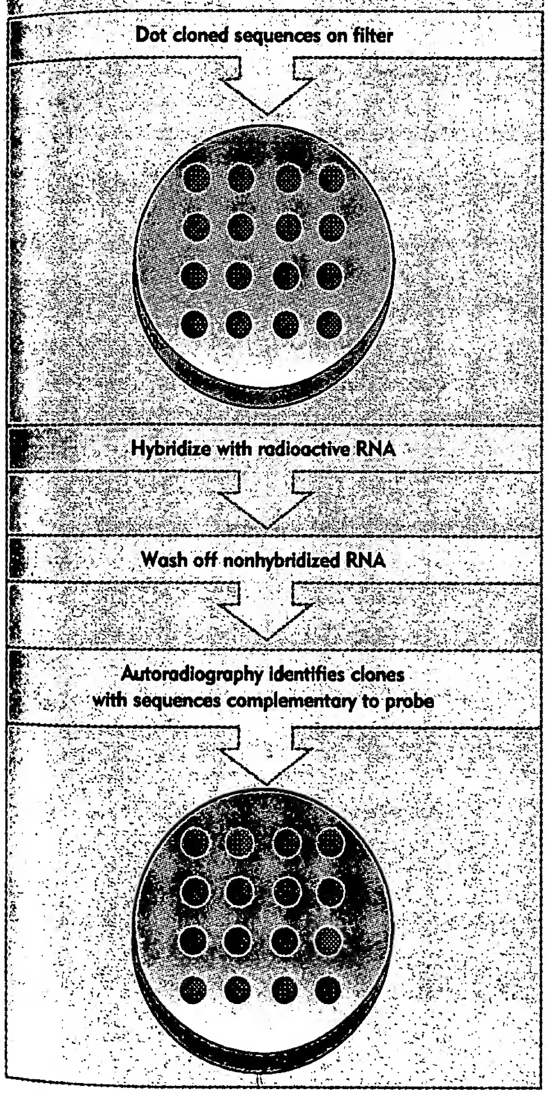
The technique can also be performed with RNA. To blot RNA from agarose onto a medium suitable for hybridization, some changes in the technique are necessary. The procedure then is known as **Northern blotting**. (And an analogous procedure that is used for transferring proteins is called **Western blotting**.)

Sometimes we want to identify the sequence in a population that corresponds to a particular probe. A variant of the blotting techniques that is useful for this purpose is called **dot blotting**. As illustrated in **Figure 21.8**, cloned DNAs to be tested (for example, representing fragments of a genome) are spotted adjacent to one another on a filter. The filter is then hybridized with a radioactively labeled probe representing a target sequence. If the sequence is represented in a particular clone, the dot representing that clone will light up by autoradiography. The intensity of the dot corresponds fairly well with the extent to which the probe is represented in the clone.

An interesting technique for isolating a particular mRNA can be used when we have at our disposal two cell types, one of which expresses the RNA and the other of which does not. The protocol used for **subtractive hybridization** is illustrated in **Figure 21.9**. The mRNA of the target cell line

Figure 21.8

Dot blotting allows many cloned sequences to be tested readily for their homology with a labeled probe. The cloned sequences are spotted on a filter paper, which is hybridized with a solution containing the labeled probe. Those spots containing sequences complementary to the probe show up by autoradiography.



is used as substrate to prepare a set of cDNA molecules corresponding to all the expressed genes. To remove sequences that are not specific for the target cell, the cDNA preparation is exhaustively hybridized with the mRNA of another, closely related cell. This step removes all the sequences from the cDNA preparation that are common to the two cell types. So the specificity of the technique will depend on the closeness of the relationship between the two cells. After discarding all the cDNA sequences that hybridize with the other mRNA, those that are left (<5% if the technique works well) are hybridized with mRNA from the target cell to confirm that they represent coding sequences. These clones should contain sequences specific to the mRNA population of the target cell, and they can then be characterized.

This technique was used to isolate clones corresponding to the T cell receptor, a protein expressed in T lymphocytes, but not in the closely related B lymphocytes that could therefore be used to provide the subtracting mRNA. Of the original cDNA preparation from T cells, 2.6% was left at the end of the procedure. It led to the isolation of 7 clones, one of which proved to be the specific T cell gene that was sought. The number of clones isolated from this approach tends to be small, but it has proved effective in several cases of this type.

A powerful technique for directly amplifying short segments of the genome is provided by the **polymerase chain reaction (PCR)**. It requires that we know the sequence on either side of the target region, and allows the region between two defined sites to be amplified.

Figure 21.10 summarizes the protocol. A preparation of DNA (usually just an extract of the whole genome) is denatured. The single-stranded preparation is annealed with two short primer sequences (~20 bases each) that are complementary to sites on the opposite strands on either side of the target region. DNA polymerase is used to synthesize a single strand from the 3′-OH end of each primer. The entire cycle can then be repeated by denaturing the preparation and starting again. The number of copies of the target sequence in principle grows exponentially. In practice, it doubles with each cycle until reaching a plateau at which more primer–template accumulates than the